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The influence of tetracyclines on T cell activation

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SUMMARY

Minocycline has been shown to have an anti-inflammatory effect in patients with rheumatoid arthritis (RA). Since there is evidence that RA is a T cell-mediated disease, we investigated the effect of minocycline on human T cell clones derived from the synovium of an RA patient. The T cells, when activated via the T cell receptor (TCR)/CD3 complex, were suppressed functionally by minocycline, resulting in a dose-dependent inhibition of T cell proliferation and reduction in production of IL-2, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). Besides an inhibition of IL-2 production, minocycline exerted its effect on T cell proliferation by induction of a decreased IL-2 responsiveness. We showed that the chelating capacity of minocycline plays a crucial role in the inhibitory effect on T cell function, since the inhibitory effect on T cell proliferation could be annulled by addition of exogenous Ca^{2+}. However, minocycline did not markedly influence the typical TCR/CD3-induced intracellular Ca^{2+} mobilization. Taken together, the results clearly indicate that minocycline has immunomodulating effects on human T cells.

Keywords tetracyclines minocycline T cell function immunosuppression

INTRODUCTION

Tetracyclines are reported to have an ameliorative effect in arthritis in animals. Minocycline, a tetracycline derivative, decreased the incidence and severity in the collagen and adjuvant models of chronic arthritis in rats [1]. Additionally CMT, a chemically modified tetracycline without antibacterial activity, in combination with a non-steroidal anti-inflammatory drug, prevented bone loss in rats with adjuvant arthritis [2].

Rheumatoid arthritis (RA) is a chronic joint disease in man. In the pathogenesis of RA, T cells are held responsible for the initiation and perpetuation of ongoing synovial inflammation (for review, see [2]), which may lead to cartilage and joint destruction brought about by enzymes, especially matrix metalloproteinases.

Two open clinical studies have suggested a beneficial effect of minocycline in patients with RA [4,5]. In a double-blind placebo-controlled trial minocycline suppressed the laboratory parameters of disease activity, especially the acute-phase reactants, although the effects on clinical signs and symptoms were less pronounced [6].

Tetracyclines are predominantly known for their antimicrobial features. However, two other properties of minocycline should be considered to account for the observed phenomena in patients treated with minocycline: tetracyclines inhibit the activity of matrix metalloproteinases [7], and are immunomodulatory. In animals, tetracyclines have been shown to suppress the DTH, the rejection of transplants [8–11] and levels of serum immunoglobulin [9,12]. Furthermore, *in vitro* tetracyclines inhibited the proliferative response of human peripheral blood mononuclear cells (PBMC) to mitogens [13–16].

We studied the effect of minocycline on T cell activation, in particular in T cell clones derived from a patient with RA. In the present study, the T cells were activated by triggering the T cell receptor (TCR)/CD3 complex, which results in the generation of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Subsequently, IP_3 induces a rapid rise of intracellular free calcium ($[Ca^{2+}]_i$), whereas DAG activates protein kinase C, which together lead to the synthesis of IL-2, an autocrine growth factor of T cell proliferation. The effect of minocycline on several aspects of T cell activation were studied in more detail.

MATERIALS AND METHODS

Reagents

Minocycline HCl, kindly provided by D. G. Lederle (Etten-Leur, The Netherlands), and doxycycline HCl, kindly provided by Pfizer (Capelle a/d IJssel, The Netherlands) were obtained in

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powder form. Minocycline HCl was dissolved in culture medium (Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml; Boehringer Mannheim, Mannheim, Germany), and fetal calf serum (FCS, 10%; GIBCO)) at a concentration of 100 μg/ml. Doxycycline HCl was dissolved in 0·02 N HCl at a concentration of 1 mg/ml and further diluted in culture medium. The antibiotic solutions were freshly prepared on the day of use and diluted to the appropriate concentrations. The concentrations used in the experiments ranged from 0·8 to 50 μg/ml. Anti-CD3 MoAbs (OKT3) were obtained from Cilag (Herenthals, Belgium). Recombinant (r) IL-2 was obtained from Cetus (Gouda, The Netherlands). Indo-1/AM was obtained from Molecular Probes (Junction City, OR).

Cell cultures

T cell clones (two $CD4^+$ (N16, N18) and one $CD8^+$ (N17)) were derived from the synovium of a patient with rheumatoid factor-positive classical RA. The cloning procedure has been described in detail [17]. In short, fragments of synovial tissue were placed in 24-well tissue culture plates (Costar 3524; Cambridge, MA) in culture medium and 5% v/v T cell growth factor (TCGF). After approximately 1 week, growing cells were separated from the tissue fragments and cloned by limiting dilution in a feeder cell mixture consisting of irradiated (30 Gy) allogeneic PBMC (10^6/ml), OKT3 ascites (10^{-5} dilution), and 5% v/v TCGF. Clones were expanded and restimulated every 7 days with a feeder cell mixture. In between the clones were maintained in culture in the presence of 5% v/v TCGF.

Proliferation experiments

Overnight 96-well tissue culture plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated at room temperature with anti-CD3 MoAb (0·1 μg/ml; 0·1 ml/well) diluted in PBS. Before use the plates were washed three times with PBS. Cloned T cells were washed free of TCGF and plated at a concentration of 2×10^5 cells/well. The cells were incubated in the presence or absence of the drug for 48 h at 37°C, 5% CO_2. Twenty-four hours after initiation of the experiment, ^{3}H-thymidine (20 μCi/ml; 0·05 ml/well) was added. The cells were harvested with a Skatron AS semiautomatic cell harvester on filter paper discs. The rate of DNA synthesis was measured by liquid scintillation counting of the air-dried discs in a 1217 Rackbeta liquid scintillation counter. Experiments were performed in triplicate.

Cytokine production experiments

Washed cloned T cells (1×10^6 or 2×10^6 cells/well) were stimulated with plastic-immobilized anti-CD3 MoAb (0·1 μg/ml; 0·5 ml/well) in 24-well tissue culture plates with or without the drug for 24 h at 37°C, 5% CO_2. Cell-free culture supernatants were stored at −20°C.

Interferon-gamma assay

For the determination of interferon-gamma (IFN-γ) an ELISA [18] (kindly provided by Dr P. Van der Meide, Rijswijk, The Netherlands) was used. Briefly, microtitre plates were coated with anti-IFN-γ MoAb (MD2) for 3 h at 37°C. The test samples and rIFN-γ standards were incubated overnight at room temperature. The next day biotinylated anti-IFN-γ MoAb (MD1) was added, followed by the addition of streptavidin conjugated to horseradish peroxidase (HRP; Zymed, Sanbio, Uden, The Netherlands). For developing the assay, orthophenylenediamine (OPD; Sigma, St Louis, MO) in phosphate buffer pH 5·6, activated with H_2O_2, was used. The reaction was stopped with 10% H_2SO_4. All reagents and samples were diluted in PBS with 0·05% Tween (PBS–T) and 1% new born calf serum (NBCS) and between the incubation steps plates were washed three times with PBS–T. Optical densities (OD) were read in a Titertek Multiscan Plus at 492 nm. The sensitivity was 0·2 ng/ml.

Tumour necrosis factor-alpha assay

For the determination of tumour necrosis factor-alpha (TNF-α) an ELISA [19] (kindly provided by Dr W. Buurman, Maastricht, The Netherlands) was used. Briefly, immuno-Maxisorb plates were coated overnight at 4°C with 0·125 μg/well of the anti-human TNF-α MoAb 61E71 in PBS. Non-specific binding was prevented by incubation of the plates for 1 h at room temperature with bovine serum albumin (BSA; Organon Teknika, Oss, The Netherlands) 1% (w/v) in PBS. The test samples and rTNF-α standards were incubated for 2·5 h at room temperature. Subsequently, the plates were incubated with a rabbit polyclonal anti-human TNF-α immunoglobulin, followed by a peroxidase-conjugated goat anti-rabbit IgG. Next, the plates were washed three times with distilled water containing 0·1% Tween, and developed with ABTS activated with H_2O_2. The reaction was stopped with 2% oxalate acid. Reagents and samples were diluted in PBS–0·1% BSA (w/v). Finally, OD were read at 405 nm, using a Titertek Multiscan Plus. The sensitivity was 10 pg/ml.

IL-2 assay

IL-2 was determined by an ELISA (Boehringer Mannheim), according to the manufacturer's description. The sensitivity of the ELISA was 62·5 pg/ml.

Determination of IL-2 receptor

For determination of IL-2R (CD25) expression flow cytometric analysis was used. Per sample, 2×10^5 cells were incubated with anti-Tac MoAb (the hybridoma producing anti-Tac was obtained from the American Type Culture Collection (ATCC, Rockville, MD)) for at least 30 min on ice. After two washes with ice-cold PBS containing 1% (w/v) BSA, cells were incubated with FITC-conjugated goat anti-mouse IgG (Nordic Immunological Labs, Tilburg, The Netherlands) diluted 1:40 in PBS–1% BSA. Cells were washed twice and analysed on a FACStar (Becton Dickinson, Mountain View, CA). The experiment was performed in duplicate on two separate occasions.

Measurement of intracellular free calcium

$[Ca^{2+}]_i$ was measured as described before [20]. In short, prewarmed Jurkat cells (5 min at 37°C, 10×10^6/ml) were resuspended in HEPES medium (NaCl (133 mM), KCl (6 mM), $MgSO_4$ (1 mM), $CaCl_2$ (1 mM), potassium phosphate (1 mM), glucose (5·5 mM), HEPES (20 mM), and human albumin (0·5% w/v), pH 7·4) and loaded with 1 μM Indo-1/AM during 45 min at 37°C. Subsequently, the cells were washed, resuspended at 10×10^6 cells/ml in the HEPES medium and kept at room temperature. Fluorescence measurements were performed at

37°C in stirred suspensions after dilution of the cells to 1 × 10^6/ml. Calibration of Indo-1 fluorescence as a function of $[Ca^{2+}]_i$ was determined as follows: to saturate all trapped Indo-1 with Ca^{2+}, 5 μM digitonin was added to the cell suspension. Subsequently, the Indo-1 signal was quenched by adding 0·5 mM Mn^{2+}. $[Ca^{2+}]_i$ was calculated as described [21], using 250 nM as the K_d for the Indo-1/Ca^{2+} complex.

Viability

Cell viability was determined by trypan blue exclusion and measurement of lactate dehydrogenase activity and the concentration of β_2-microglobulin in the supernatant. Lactate dehydrogenase activity was determined on a BM/Hitachi 747 (Boehringer Mannheim, Almere, The Netherlands) using an ultraviolet method according to the manufacturer's instructions. Levels of β_2-microglobulin were determined by IMx β_2-microglobulin assay (Abbott Labs, Santa Clara, CA), which is a microparticle enzyme immunoassay. The assay was performed according to the manufacturer's instructions. The sensitivity of the test is 5 μg/*l*.

Statistical analysis

The effect of the drug was expressed as percentage inhibition, which is defined as (1 − (effect in the presence of the drug/effect in the absence of the drug)) × 100%.

Non-parametric tests were used to determine statistically significant differences.

The correlation coefficient between the effects of the different doses of minocycline and doxycycline was estimated by means of the intraclass correlation coefficient. This was calculated using multivariate analysis of variance (MANOVA) with dose as a within-subjects factor.

RESULTS

Minocycline inhibits TCR/CD3-induced proliferation in $CD4^+$ and $CD8^+$ synovial T cell clones

In one $CD8^+$ (N17) and two $CD4^+$ (N16, N18) synovial T cell clones proliferation was induced with plastic-immobilized anti-CD3 MoAb in the presence or absence of minocycline. Minocycline inhibited the proliferation of each T cell clone in a dose-response manner (Fig. 1).

Cell viability, as measured with trypan blue staining, was >80% with all drug concentrations tested. Accordingly, no significant differences in lactate dehydrogenase release could be measured in the presence of minocycline at concentrations of 25 and 50 μg/ml. Moreover, the production of the constitutively expressed protein β_2-microglobulin was measured to investigate the viability of the cells. In unstimulated T cells (1 × 10^6 cells) the total β_2-microglobulin concentration was 85 ± 27 ng/ml (mean ± s.d.). A slight increase in β_2-microglobulin production to 111 ± 32 ng/ml was observed when T cells were stimulated via the TCR/CD3 complex for 24 h; in the presence of 25 μg/ml minocycline the production of β_2-microglobulin, being 94 ± 26 ng/ml, was always more than the amount of β_2-microglobulin produced by resting T cells.

Since minocycline affected all three clones to the same extent, we continued our experiments with one representative clone (N18).

Minocycline inhibits TCR/CD3-induced IL-2, IFN-γ and TNF-α production by a $CD4^+$ synovial T cell clone

Stimulation of the $CD4^+$ T cell clone N18 by anti-CD3 MoAb coated to tissue culture plates induced the production of IL-2, IFN-γ and TNF-α. Minocycline inhibited IL-2 production in a dose-dependent manner. Minocycline at a concentration of 25 μg/ml inhibited IL-2 production 26 ± 7·5% (mean ± s.e.m.) (Fig. 2). Minocycline also inhibited IFN-γ and TNF-α production in a dose-response manner. The maximal percentage inhibition was 23·9 ± 3·1% and 21·5 ± 5% for IFN-γ and TNF-α production, respectively, at a concentration of 25 μg/ml minocycline (Fig. 2).

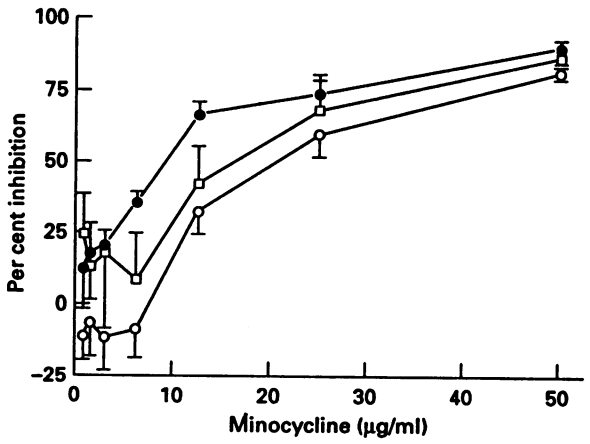

Fig. 1. The effect of minocycline on anti-CD3-induced proliferation of synovial T cell clones (○, N16; □, N17; ●, N18). The symbols reflect mean ± s.e.m. of at least three experiments. The results are presented as percentage inhibition of control in the absence of minocycline. The control ranged from 12 351 to 45 729, from 17 132 to 72 985, and from 10 118 to 64 794 ct/min for N16, N17 and N18, respectively.

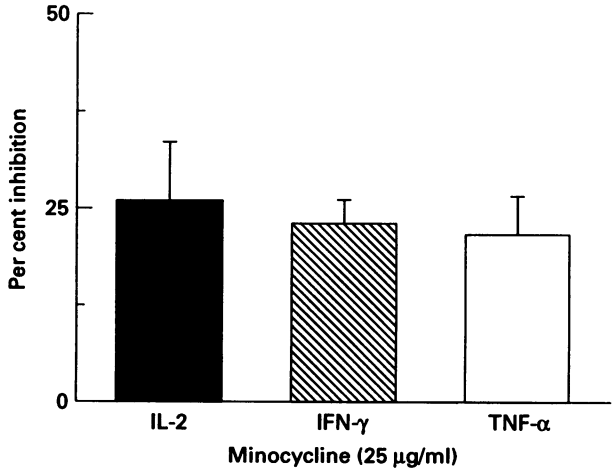

Fig. 2. The effect of minocycline on anti-CD3-induced IFN-γ and tumour necrosis factor-alpha (TNF-α) production by cloned T cells (N18). Shown are mean ± s.e.m. of three, five and six experiments for IL-2, IFN-γ and TNF-α, respectively, in the presence of 25 μg/ml minocycline. The results are described as percentage inhibition of induced production in the absence of minocycline. The production of the individual cytokines ranged from 625 to 7820 pg/ml for IL-2, from 92 to 1197 ng/ml IFN-γ, and from 7 to 40 ng/ml for TNF-α.

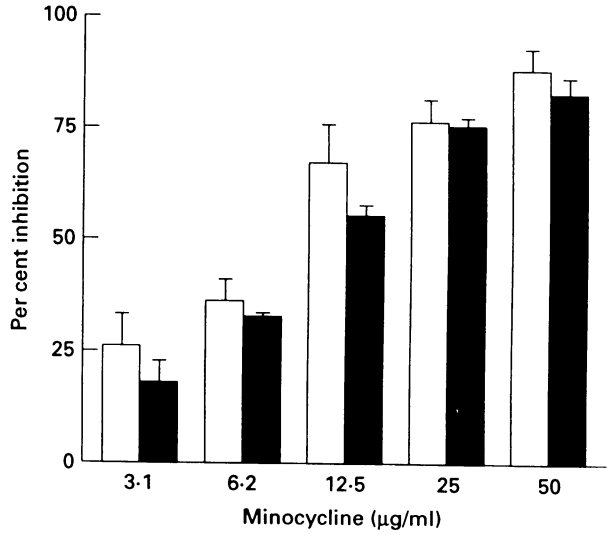

Fig. 3. Inhibition of anti-CD3-induced proliferation in cloned T cells (N18) by minocycline could not be restored by the addition of exogenous recombinant (r)IL-2. Shown are mean ± s.e.m. of three experiments. The results are described as percentage inhibition of control. The control ranged from 10 903 to 68 458 and from 16 158 to 56 641 ct/min for 0 and 10 U/ml IL-2, respectively. □ and ■ represent, respectively, addition of 0 and 10 U/ml rIL 2.

Minocycline inhibits IL-2 responsiveness in synovial T cell clones

Next, we determined whether the inhibitory effect of minocycline on T cell proliferation could be bypassed by the addition of IL-2. The $CD4^+$ T cell clone is known to express IL-2R and to respond to IL-2 addition by proliferation. However, the addition of rIL-2 (0·1, 1 or 10 U/ml) did not bypass the inhibitory effect of minocycline on TCR/CD3-induced proliferation in cloned T cells (Fig. 3). In proliferation experiments in which cloned T cells were stimulated with rIL-2 (20 U/ml) in the absence or presence of minocycline, an inhibition of T cell proliferation by minocycline in a dose-dependent manner was seen (Fig. 4).

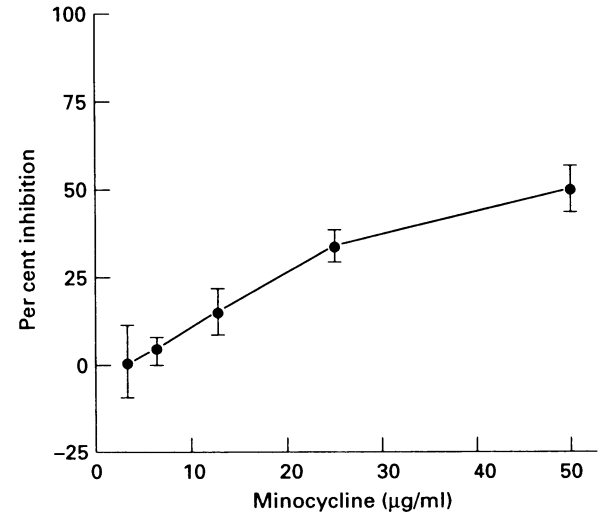

Fig. 4. The effect of minocycline on IL-2-induced proliferation in cloned T cells (N18). The symbols represent mean ± s.e.m. of three experiments. The results are described as percentage inhibition of control. The control ranged from 44 385 to 75 895 ct/min.

To investigate whether inhibition of IL-2 responsiveness was caused by down-regulation of the expression of the IL-2R α chain (CD25) by minocycline, cloned synovial T cells were stimulated with anti-CD3 MoAb in the presence or absence of minocycline for 24 h and subsequently analysed for IL-2R expression using anti-Tac MoAb. In the absence of minocycline 86–92% of T cells were positive for IL-2R (mean fluorescence intensity ranging from 518 to 544) against 86–96% in the presence of 50 μg/ml minocycline (mean fluorescence intensity ranging from 437 to 542).

Doxycycline, like minocycline, inhibits TCR/CD3-induced T cell proliferation and IFN-γ production

To study whether the effect on T cell activation was a more general property of tetracyclines, another tetracycline derivative, i.e. doxycycline, was studied also. Doxycycline and minocycline have corresponding biochemical characteristics with regard to lipid solubility and chelating capacity.

Both drugs inhibited anti-CD3-induced proliferation in a concentration-dependent way and to the same extent (MANOVA, $r = 0{\cdot}65$; $P = 0{\cdot}064$) (Fig. 5a). The inhibitory capacity of doxycycline and minocycline towards TCR/CD3-induced IFN-γ production was similar (MANOVA, $r = 0{\cdot}71$; $P = 0{\cdot}001$) (Fig. 5b). Similar to minocyclin we observed no toxic effects of doxycycline on T cell functioning in the presence of the concentrations tested.

The inhibitory effect of minocycline on proliferation is partially reversible

When cloned synovial T cells were preincubated with minocycline at a concentration of 50 or 12·5 μg/ml for 24 h at 37°C, 5% CO_2, subsequently harvested, washed, counted, adjusted to cell number and finally incubated on plates coated with anti-CD3 MoAb, we also observed inhibition of T cell proliferation. However, the inhibitory effect of preincubation with 50 or 12·5 μg/ml minocycline was less, namely 30% and 19%, than the inhibitory effect of direct incubation with minocycline, being 76% and 35% (Fig. 6).

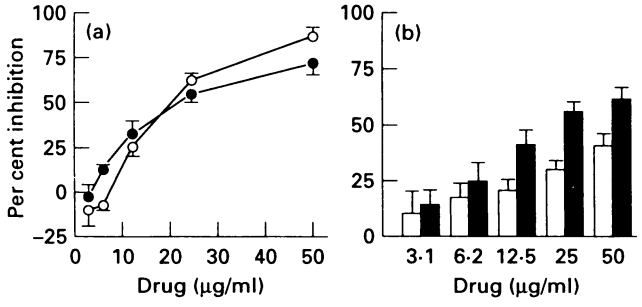

Fig. 5. The effect of doxycycline and minocycline on anti-CD3-induced proliferation (a) and IFN-γ production (b) by cloned T cells (N18). The symbols reflect the mean ± s.e.m. values of three and five separate experiments for proliferation and IFN-γ production, respectively. The results are described as percentage inhibition of control. The control values for the proliferation ranged from 17 122 to 44 431 ct/min and for IFN-γ production from 220 to 1560 ng/ml. ○, □, Minocycline; ●, ■, doxycycline.

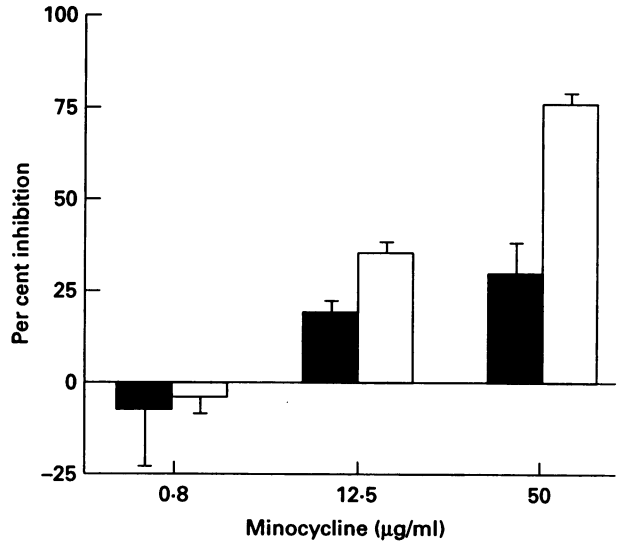

Fig. 6. The inhibitory effect of minocycline on T cell proliferation was partially reversible. Cloned T cells (N18) were preincubated with or without minocycline for 24 h at 37°C, 5% CO_2. Afterwards the cells were harvested, washed, counted, adjusted to cell number, and proliferation was induced with anti-CD3 MoAb (0·1 μg/ml). As controls, cells which had been preincubated without minocycline were incubated with minocycline. Symbols represent mean ± s.e.m. of four separate experiments. Results are described as percentage inhibition relative to proliferation in the absence of minocycline, which ranged from 12 976 to 40 040 ct/min. □, Proliferation experiments in the presence of minocycline; ■, proliferation experiments after preincubation with minocycline.

Addition of Ca^{2+} restores the inhibition of TCR/CD3-induced proliferation by minocycline

Since minocycline is a compound with chelating capacity [22], we studied whether Ca^{2+} plays a role in the inhibition of TCR/CD3-induced proliferation by minocycline.

Therefore, exogenous Ca^{2+}, ranging from 1·6 to 9·6 mM, was added to cloned synovial T cells, stimulated with plastic-immobilized anti-CD3 MoAb in the absence or presence of minocycline. The inhibitory effect of minocycline was partially abolished at low concentrations of Ca^{2+} in the culture medium and totally abolished at high concentrations of Ca^{2+} in the culture medium (Fig. 7a,b).

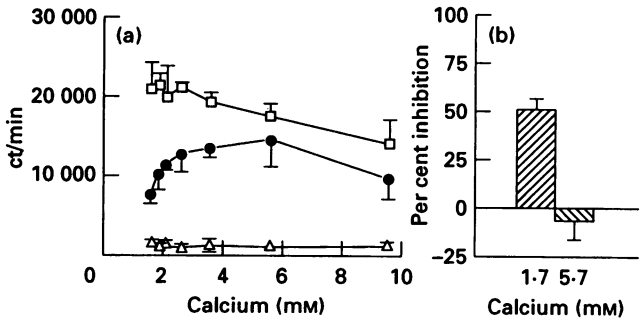

Fig. 7. Addition of Ca^{2+} restored the inhibition of anti-CD3-induced proliferation by minocycline. (a) Ca^{2+} was added in proliferation experiments of cloned T cells (N18). (△, Proliferation in medium without anti-CD3 stimulus; □, proliferation induced with plastic-immobilized anti-CD3 (0·1 μg/ml); ●, anti-CD3-induced proliferation in the presence of 25 μg/ml minocycline.) (b) Percentage inhibition by 25 μg/ml minocycline compared with control in the presence of a low and high concentration of Ca^{2+} in the culture medium. The results (mean ± s.e.m.) of four independent proliferation experiments are demonstrated.

Minocycline does not affect TCR/CD3-induced rises in $[Ca^{2+}]_i$ in Jurkat cells

To study whether minocycline affected $[Ca^{2+}]_i$ mobilization upon TCR/CD3 triggering, we studied Jurkat T cells, which are generally used as a model for resting T cells and particularly for studying $[Ca^{2+}]_i$ mobilization [23]. In a series of experiments (n = 10) on three separate occasions the basal $[Ca^{2+}]_i$ of 58 ± 24 nM increased after anti-CD3 stimulation to 222 ± 186 nM, followed by a plateau of 93 ± 26 nM. When minocycline (25 μg/ml) was present in the experiments (n = 9) the basal $[Ca^{2+}]_i$ was 115 ± 31 nM, increased to 222 ± 136 nM, and was followed by a plateau of 168 ± 75 nM. Minocycline did not influence the typical $[Ca^{2+}]_i$ response, but increased the basal and plateau levels of $[Ca^{2+}]_i$ ($P < 0·05$).

DISCUSSION

The present study shows that minocycline exerts immunomodulatory effects on human T cells.

Tetracyclines have apart from their immunomodulatory features, other properties that validate their usage as a treatment modality in RA. For instance, tetracyclines inhibit the enzyme activity of metalloproteinases [7], including synovial collagenases [24]. Therefore, cartilage and bone breakdown may be decreased as a result of treatment with tetracyclines. Concordantly, prophylactic treatment with oral doxycycline reduced the severity of canine osteoarthritis [25]. In addition, tetracyclines inhibit the activity of phospholipase A_2 [26], suppress the function of neutrophils [27,28] and scavenge oxygen radicals [29], which may result in an anti-inflammatory effect. Diminishing of joint destruction and inflammation may have a beneficial effect in RA.

In the literature, tetracyclines have been shown to inhibit the proliferative response of human T cells in monocyte-dependent, mitogen-stimulated assay systems [13–16]. Whether or not minocycline directly affects T cell proliferation *per se* is difficult to conclude on the basis of these studies in complex cell systems, especially since tetracyclines have been reported to influence monocyte functioning [30]. The present study investigated the effect of minocycline on T cells selectively and showed that minocycline affected both TCR/CD3 as well as IL-2-induced proliferation, which was due to a negative effect on IL-2 production and on IL-2 responsiveness. Besides an inhibitory effect on IL-2 production, minocycline also negatively influenced the production of the other early induced cytokine gene products IFN-γ and TNF-α [31]. Similar results were obtained with peripheral blood T cells, whereas minocycline exerted stimulatory effects on cytokine production by monocytes (Kloppenburg and Verweij, unpublised observations). These findings indicate that minocycline affects cell function in a cell-specific manner.

Since T cell-targeted therapy, including thoracic duct drainage, total lymph node irradiation, lymphocytapheresis and cyclosporin A, exerts beneficial effects in RA, compounds with immunosuppressive properties are generally considered to

be interesting candidates for treatment in RA. However, despite the effect of minocycline on T cell function *in vitro*, the effect of minocycline treatment in patients with RA was not very strong [5]. That not all T cell-targeted therapies lead to a beneficial effect in RA has also been observed in recent work of our group on the treatment of early RA with CD4 MoAbs in a placebo-controlled trial [32,33]. Despite a decline in the number of circulating $CD4^+$ cells and in histological scores for CD3, CD4, CD45RO, CD45RA, and CD38, no clinical improvement has been seen.

Tetracyclines are chelators of divalent cations [23], which may account for several features of tetracyclines. Addition of divalent cations (Ca^{2+}) *in vitro* overcomes the inhibition of metalloproteinase activity [7] and the suppression of neutrophil functions [29,34] by tetracyclines. In the present study we show that the inhibitory effect of minocycline on T cell proliferation could be overcome by addition of Ca^{2+}. The inhibitory effect was found to be higher in the continued presence of minocycline than after preincubation with the drug. Together, these findings suggest that the chelating activity of minocycline is important for the inhibition of T cell proliferation. The chelating effect of tetracyclines may possibly interfere with T cell proliferation in two ways. First, tetracylines have been shown to bind to DNA by calcium bridges [35], which may account for the inhibition of T cell proliferation. Moreover, in the association curve of DNA and tetracyclines an optimum is observed for the amount of calcium that binds to DNA [35]. Possibly, the addition of extra calcium changed the composition of the calcium–tetracycline complexes in such a way that these complexes bound less efficiently to DNA and therefore did not induce inhibition of T cell proliferation. Second, minocycline may interfere with early signal transduction, since minocycline has been shown to negatively influence the early activated genes upon TCR/CD3 stimulation (Fig. 2). Triggering of the TCR/CD3 complex results in an increased $[Ca^{2+}]_i$ which is obligatory for T cell proliferation [23,36]. The chelating capacity of minocycline may prevent the increase in $[Ca^{2+}]_i$. However, our results have demonstrated no inhibitory effect of minocycline on $[Ca^{2+}]_i$ mobilization following anti-CD3-induced T cell activation, but in contrast have shown an increase in the value of basal $[Ca^{2+}]_i$. On the other hand, these results agree with an *in vitro* study in osteoclasts showing a transient elevation of $[Ca^{2+}]_i$ after application of minocycline [37]. Accordingly, T cells from rats, treated orally with minocycline, have shown an increase in $[Ca^{2+}]_i$ upon concanavalin A stimulation *in vitro* compared with T cells from untreated rats [38].

In the present study minocycline has been shown to affect the IL-2 responsiveness of T cell clones. In the literature minocycline has been reported to inhibit the mitogen-induced up-regulation of IL-2R in peripheral T cells [16]. Synovial T cell clones, used in the present study, express IL-2R constitutively, and minocycline did not down-regulate the expression of these receptors. Thus, a diminished IL-2R expression can not explain the observed inhibition of proliferation.

In general, prediction of *in vivo* potency of drugs on the basis of *in vitro* results is limited. Serum concentrations of minocycline after a normal oral dose regimen (initial oral dose of 200 mg followed by 100 mg every 12 h thereafter) ranged from 2·3 to 3·5 μg/ml [39]. T cell function *in vitro* was found to be inhibited at a concentration of 12·5 μg/ml minocycline or higher. Since minocycline has a high degree of lipid solubility, this compound is widely distributed in the body. This results in tissue concentrations that exceed serum concentrations [40]. Moreover, lipid-soluble tetracyclines have been reported to accumulate in leucocytes [9,41,42]. In agreement with the latter are the *in vitro* studies that demonstrated that lipid-soluble tetracyclines are more potent inhibitors of mitogen-induced proliferation than non-lipid-soluble ones [13–16].

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REFERENCES

1 Breedveld FC, Trentham DE. Suppression of collagen and adjuvant arthrtis by a tetracycline. Arthritis Rheum 1988; **13** (Suppl):R9.
2 Panayi GS, Lanchbury JS, Kingsley GH. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. Arthritis Rheum 1992; **35**:729.
3 Greenwald RA, Moak SA, Ramamurthy NS, Golub LM. Tetracyclines suppress matrix metalloproteinase activity in adjuvant arthritis and in combination with flurbiprofen, ameliorate bone damage. J Rheumatol 1992; **19**:927.
4 Breedveld FC, Dijkmans BAC, Mattie H. Minocycline treatment for rheumatoid arthritis: an open dose finding study. J Rheumatol 1990; **17**:14.
5 Langevitz P, Zemer D, Book M, Pras M. Treatment of resistant rheumatoid arthritis with minocycline: an open dose study. J Rheumatol 1992; **19**:1502.
6 Kloppenburg M, Breedveld FC, Terwiel JPh., Mallée C, Dijkmans BAC. Minocycline in active rheumatoid arthritis (RA): a double-blind placebo-controlled trial. Arthritis Rheum 1994; **37**:629.
7 Golub LM, Lee HM, Lehrer G, Nemiroff A, McNamara TF, Kaplan R, Ramamurthy NS. Minocycline reduces gingival collagenolytic activity during diabetes. J Periodont Res 1983; **18**:516.
8 Thong YH, Ferrante A. Effect of tetracycline treatment on immunological responses in mice. Clin Exp Immunol 1980; **39**:728.
9 Van den Bogert C, Kroon AM. Effects of oxytetracycline on *in vivo* proliferation and differentiation of erythroid and lymphoid cells in the rat. Clin Exp Immunol 1982; **50**:327.
10 Bellahsene A, Forsgren A. Effect of doxycycline on immune response in mice. Infect Immun 1985; **48**:556.
11 Rijkers GT, Teunissen AG, Van Oosterom R, Van Muiswinkel WB. The immune system of cyprinid fish. The immunosuppressive effect of the antiobiotic oxytetracycline in carp. Aquaculture 1980; **19**:177.
12 Rijkers GT, Van Oosterom R, Van Muiswinkel WB. The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp. Vet Immunol Immunopathol 1981; **2**:281.
13 Thong YH, Ferrante A. Inhibition of mitogen-induced human lymphocyte proliferative responses by tetracycline analogues. Clin Exp Immunol 1979; **35**:443.
14 Banck G, Forsgren A. Antibiotics and suppression of lymphocyte function *in vitro*. Antimicrob Agents Chemoth 1979; **16**:554.
15 Potts RC, Hassan HAA, Brown RA, MacConnacie A, Gibbs JH, Robertson AJ, Swanson Beck J. *In vitro* effects of doxycycline and tetracycline on mitogen stimulated lymphocyte growth. Clin Exp Immunol 1983; **53**:458.
16 Ingham E, Turnbull L, Kearney JN. The effects of minocycline and tetracycline on the mitotic response of human peripheral blood lymphocytes. J Antimicrob Chemother 1991; **27**:607.

17 Miltenburg AMM, Van Laar JM, De Kuiper R, Daha MR, Breedveld FC. T cells cloned from human rheumatoid synovial membrane functionally represent the Th1 subset. Scand J Immunol 1992; **35**:603.
18 Van der Meide PH, Dubbeld M, Schellekens H. Monoclonal antibodies to human interferon and their use in a sensitive solid-phase ELISA. J Immunol Methods 1985; **79**:293.
19 Engelberts I, Möller A, Schoen GJM, Van der Linden CJ, Buurman WA. Evaluation of measurement of human TNF in plasma by ELISA. Lymphokine Cytokine Res 1991; **10**:133.
20 Koenderman L, Yazdanbakhsh M, Roos D, Verhoeven AJ. Dual mechanism in priming of the chemoattractant-induced respiratory burst in human granulocytes. A Ca^{2+}-dependent and a Ca^{2+}-independent route. J Immunol 1989; **142**:623.
21 Bijsterbosch MK, Rigley KP, Klaus GGB. Cross-linking of surface immunoglobulin on B lymphocytes induces both intracellular Ca^{2+} release and Ca^{2+} influx: analysis with indo-1. Biochem Biophys Res Commun 1986; **137**:500.
22 Albert A, Rees CW. Avidity of the tetracyclines for the cations of metals. Nature 1956; **177**:433.
23 Gruters RA, Otto SA, Al BJM, Verhoeven AJ, Verweij CL, Van Lier RAW, Miedema F. Non-mitogenic T cell activation signals are sufficient for induction of human immunodeficiency virus transcription. Eur J Immunol 1991; **21**:167.
24 Greenwald RA, Golub LM, Lavities B, Ramamurthy NS, Gruber B, Laskin RS, McNamara TF. Tetracyclines inhibit human synovial collagenase *in vivo* and *in vitro*. J Rheumatol 1987; **14**:28.
25 Yu LP, Smith GN, Brandt KD, Myers SL, O'Connor BL, Brandt DA. Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. Arthritis Rheum 1992; **35**:1150.
26 Pruzanski W, Greenwald RA, Street IP, Laliberte F, Stefanski E, Vadas P. Inhibition of enzymatic activity of phopholipase A_2 by minocycline and doxycycline. Biochem Pharmacol 1992; **44**:1165.
27 Martin RR, Warr GA, Couch RB, Yeager H, Knight V. Effects of tetracyclines on leucotaxis. J Infect Dis 1974; **129**:110.
28 Gabler WL, Creamer HR. Suppression of neutrophil function by tetracyclines. J Periodontol Res 1991; **26**:52.
29 Van Baar HMJ, Van de Kerkhof PCM, Mier PD, Happle R. Tetracyclines are potent scavengers of superoxide radical. Brit J Dermatol 1987; **117**:131.
30 Ingham E. Modulation of the proliferative response of murine thymocytes stimulated by IL-1, and enhancement of IL-1β secretion from mononuclear phagocytes by tetracyclines. J Antimicrob Chemother 1990; **26**:61.
31 Crabtree GR. Contingent genetic regulatory events in T lymphocyte activation. Science 1989; **243**:355.
32 Van de Lubbe PA, Dijkmans BAC, Markusse HM, Nässander U, Breedveld FC. Lack of clinical effect of CD4 monoclonal antibody therapy in early rheumatoid arthritis; a placebo-controlled trial. Arthritis Rheum 1994; **37**:S294.
33 Tak PP, Van der Lubbe PA, Daha MR, Kluin PhM, Meinders AE, Breedveld FC. Reduction of synovial inflammation without clinical improvement after CD4 monoclonal antibody treatment. Arthritis Rheum 1994; **37**:S337.
34 Goodhart GL. Further evidence for the role of bivalent cations in human polymorphonuclear leucocyte locomotion: recovery from tetracycline-induced inhibition in the presence of cation ionophores. J Reticuloendothial Soc 1979; **25**:545.
35 Kohn KW. Mediation of divalent metalions in the binding of tetracycline to macromolecules. Nature 1961; **191**:1156.
36 Tsien RY, Pozzan T, Rink TJ. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. Nature 1982; **295**:68.
37 Bax CMR, Shankar VS, Towhidul Alam ASM *et al.* Tetracyclines modulate cytosolic Ca^{2+} responses in the osteoclast associated with 'Ca^{2+} receptor' activation. Bioscience Rep 1993; **13**:169.
38 Sewell KL, Furrie E, Trentham DE. The therapeutic effect of minocycline in experimental arthritis. Arthritis Rheum 1990; Suppl: S106.
39 Macdonald H, Kelly RG, Allen ES, Noble JF, Kanegis LA. Pharmacokinetic studies of minocycline in man. Clin Pharmacol Ther 1973; **14**:852.
40 Saivin S, Houin G. Clinical pharmacokinetics of doxycycline and minocycline. Clin Pharmacokin 1988; **15**:355.
41 Park JK, Dow RC. The uptake and localization of tetracycline in human blood cells. Br J Exp Pathol 1970; **51**:179.
42 Gabler WL. Fluxes and accumulation of tetracyclines by human blood cells. Res Commun Chem Path Pharm 1991; **72**:39.